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Determination of HCV Quasispecies by Cloning and Sequencing

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1. Introduction

Most RNA viruses exists as a heterogeneous mixture of closely related viral genome in the host, which is the result of high error rates in RNA replication and selected by various viral and host factors. The spectrum of this spectrum of related genomes within a host is referred as quasispecies *(1)*. This important biological characteristics of RNA virus is believed to contribute to viral persistence in the presence of host immune system and during treatment with antiviral agents. Hepatitis C virus (HCV), being a single-strand RNA virus, is no exception. In HCV genome, the most heterogeneous regions lie within the envelope 2 region, which are referred to as hypervariable regions (HVRs). The HVRs in HCV are believed to be the result of high nucleotide variation and immune selection pressure *(2)*. Because of its heterogeneity, this is a good region for the study of HCV quasispecies.

Interferon (IFN) therapy has been shown to be effective in the treatment of patients with chronic hepatitis C *(3)*. Approximately 15–30% of patients who received IFN therapy have normalization of their serum ALT levels and the elimination of HCV RNA. The remaining patients had persistent viremia and LAT elevations, or had a relapse shortly after IFN was discontinued *(4,5)*. There is evidence that certain virologic factors are associated with the outcome of IFN therapy, including HCV genotype, viremia level, and the level of heterogeneity of HCV quasispecies *(6,7)*. This chapter describes our technique of studying HCV quasispecies before and after IFN therapy using a combination of cloning and sequencing, with the sequences analyzed by molecular evolutionary analysis *(8,9)*

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2. Materials

1 Serum samples In the study described, samples were obtained from a Japanese patient with genotype 1b, five time points were studied (A 1 yr before IFN, B just before IFN, C· just before IFN, D. at the biochemical relapse time, E: 1 yr after IFN)
2 RNAzol B (Cinna/Biotecx Laboratories, Friendswood, TX)
3 Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT, Gibco-BRL, Gaithersburg, MD).
4 1X RT buffer 50 m*M* Tris-HCl, pH 8.3, 75 m*M* KCl, 3 m*M* $MgCl_2$, and 10 m*M* DDT
5 dNTP (Promega, Madison, WI)
6 Placental RNase inhibitor (RNasin, TOYOBO, Osaka, Japan)
7 Thermal cycler (Perkin-Elmer Cetus, Norwalk, CT).
8 *Taq* polymerase and reaction buffer (Promega)
9 1X PCR buffer (10 m*M* Tris-HCl, pH 9.0, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0 01% gelatin, and 0.1% Triton X-100)
10 Seakem agarose (FMC BioProducts, Rockland, ME)
11 Magic Minipreps (Promega)
12 *Bam*HI and *Eco*RI (Takara, Shiga, Japan).
13 pGEM-3zf(+) vector (Applied Biosystems, Foster City, CA).
14 DNA ligation kit (Takara, Shiga, Japan)
15 Competent cell *Escherichia coli*· DH5a (Takara)
16 373A DNA sequencer (Applied Biosystems)
17 PRISM Ready Reaction Dye Primer Cycle Sequencing Kit (Applied Biosystems)

3. Methods

1 RNA extraction. RNA is extracted from 100 μL of serum with RNAzol B The extracted RNA was precipitated with isopropanol and washed with ethanol. The RNA pellet is dissolved in diethylpyrocarbonate-treated distilled water containing 100 U of human placental ribonuclease inhibitor (RNasin).
2 Reverse transcription: The first-strand cDNA is synthesized from the extracted RNA at 37°C for 60 min using random hexamer, and with 200 U of M-MLV-RT in a 20-μL mixture containing 1X RT buffer and 600 μ*M* each dNTP. Reaction is terminated by heating at 95°C for 5 min, and mixtures are then chilled on ice
3. PCR For PCR, a 50-μL mixture containing 1X PCR buffer, 2 μL of the resulting cDNA, 1 25 U of *Taq* DNA polymerase (Promega), and primers is prepared The cDNA is amplified for 40 cycles with the following parameters: a preliminary 20 cycles of amplification with 94°C for 1 min (denaturing), 45°C for 1 min (annealing), and 72°C for 1 min (extension), followed by 20 additional cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. For the second-round PCR, two μL of the first round PCR product is amplified for another 30 cycles Each cycle is at 94°C for 1 min, 60°C for 45 s, and 72°C for 1 min The primers used are as follows (*see* **Note 1**)·

Outer primers 5'-CAG(C/T)T(A/G)CTCCGGATCCCACAAGC-3', and
5'-ACGTCCGTCTCATT(C/T)(T/G)C(A/C)CCCCA-3'

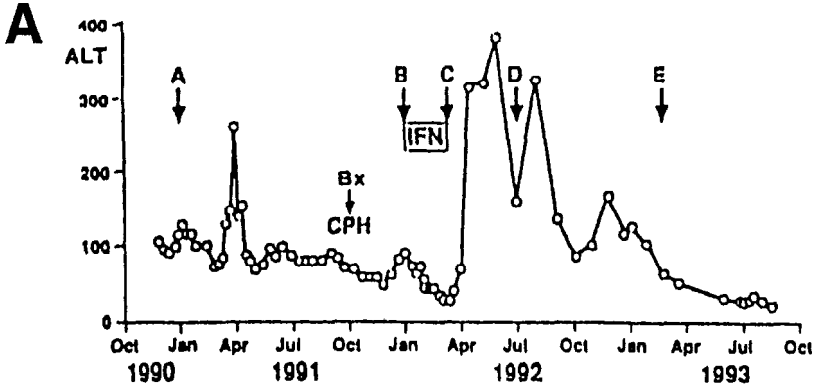

Fig. 1 **(A)** Biochemical profile of the patient and the study time points *(continued)*

Inner primer 5'-TCTGGATCCTATTCCATGGTGGGGAACTGG-3' (with a *Bam*HI site) and
5'-AATGAATTCTACAACAGGGCT(T/G)GG(A/G)GTGAA-3' (with an *Eco*RI site)

Ligation and transformation. The PCR amplicons are digested with the respective enzymes and gel-purified with Magic MiniprepsTM The pGEM-3zf(+) vector and purified PCR product are then ligated with T4 ligase at a ratio of 1 1–3 at 16°C for 30 min (as described in the DNA ligation kit) The DNA is then transformed into competent cells, the cells were spread on the LB plate, and colonies with insert are color-selected with X-gal staining (the DNA insert will disrupt the *lac*Z gene and the colonies with the vector with DNA insert should not change X-gal from colorless to blue) (*see* **Note 2**).

4 Sequencing and analysis. To avoid selection bias, at least 10 clones (in the experiment described, at time point A 11 colonies, B· 47, C 12, D: 24, and E 12 clones) are selected and their sequence determined bidirectionally with the dideoxynucleotide chain-termination method using a 373A DNA sequencer. The sequences obtained are maximally aligned, the number of nucleotide substitutions and nucleotide diversity are then estimated by the six-parameter method, and phylogenetic tree is constructed using the neighbor-joining method, as described in Chapter 12 (*see* **Note 3**)

4. Notes

1. PCR experiments are prone to contaminations. The general precautions to avoid PCR contamination must be followed
2. In ligation, transformation and sequencing, a number of potential problems may occur in every step Therefore, positive and negative controls should be employed in every step For details, refer to standard molecular biology textbooks.
3. In the experiments performed and described in this chapter, the quasispecies nature of HCV is obvious as seen in the phylogenetic tree (**Fig. 1**). All clones obtained

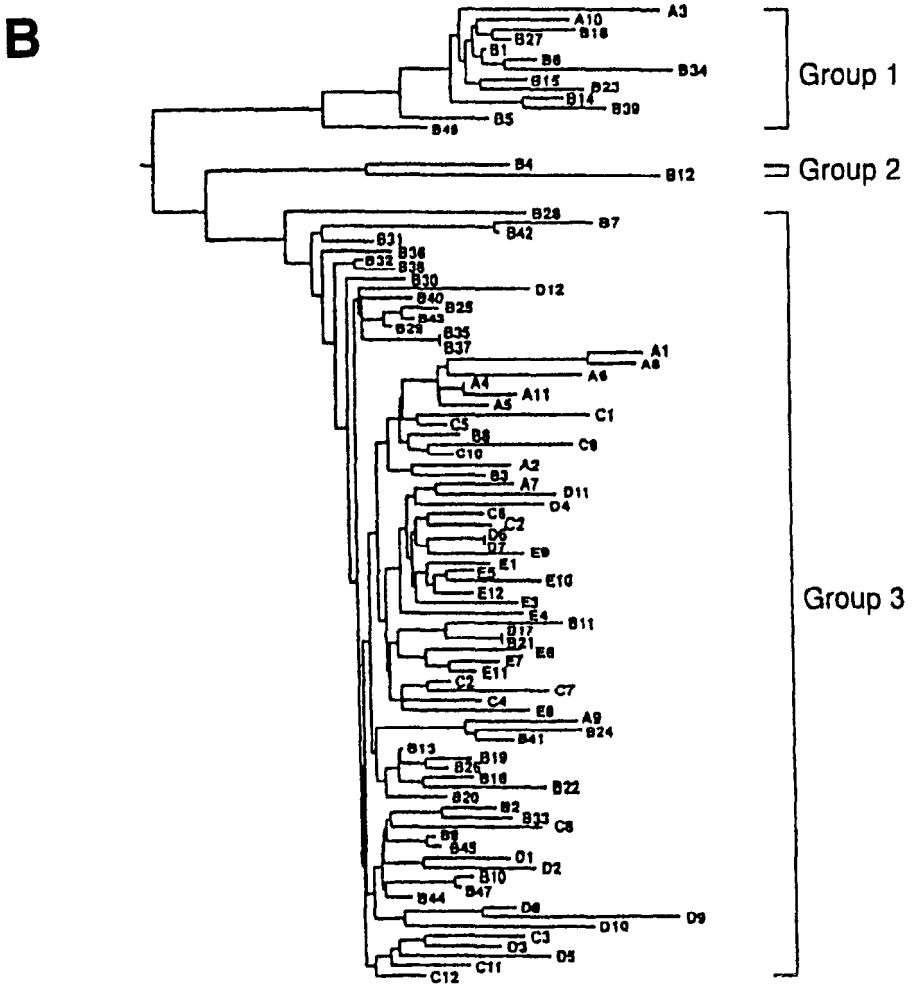

Fig. 1. **(B)** The phylogenetic tree analysis of the clones. Letters represent time points and numbers represent the clone numbers. The clones can be divided into three groups based on evolutionary distances *(9)*.

from the same time-point are referred to by the letter followed by the clone number Note that IFN therapy drifted the quasispecies equilibrium of HCV

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